MAJOR ARTICLE

Severe Acute Respiratory Syndrome Coronavirus Infection in Vaccinated Ferrets

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Background. Development of vaccines to prevent severe acute respiratory syndrome (SARS) is limited by the lack of well-characterized animal models. Previous vaccine reports have noted robust neutralizing antibody and inflammatory responses in ferrets, resulting in enhanced hepatitis.

Methods. We evaluated the humoral immune response and pathological end points in ferrets challenged with the Urbani strain of SARS-associated coronavirus (SARS-CoV) after having received formalin-inactivated whole-virus vaccine or mock vaccine.

Results. Humoral responses were observed in ferrets that received an inactivated virus vaccine. Histopathological findings in lungs showed that infection of ferrets produced residual lung lesions not seen in both mock and vaccinated ferrets. SARS-CoV infection demonstrated bronchial and bronchiolar hyperplasia and perivascular cuffing in ferret lung tissue, as seen previously in infected mice. No evidence of enhanced disease was observed in any of the ferrets. All of the ferrets cleared the virus by day 14, 1 week earlier if vaccinated.

Conclusions. The vaccine provided mild immune protection to the ferrets after challenge; however, there was no evidence of enhanced liver or lung disease induced by the inactivated whole-virus vaccine. The ferret may provide another useful model for evaluating SARS vaccine safety and efficacy.

Severe acute respiratory syndrome (SARS)–associated coronavirus (SARS-CoV) is an emerging infectious pathogen of zoonotic origin. SARS-CoV was found in certain bat populations in China [1, 2]. An outbreak occurred in China in late 2002 after the virus was apparently amplified in an intermediary host at a Chinese wet market [3]. This pandemic lasted through July 2003, with >8000 people infected worldwide and 774 deaths in 26 countries [4]. Since the end of the SARS pandemic, several infections have been observed [5]. No direct transmission from bat to human has been observed to date, but SARS has the potential to reappear from an animal reservoir or through accidental or intentional release. The development of effective vaccines and animal models is highly desirable for the prevention and containment of future outbreaks of SARS-CoV.

Attenuated and killed SARS-CoV, DNA, and viral-vectored vaccines are being evaluated in a number of animal models, including the mouse, ferret, hamster, and nonhuman primate [6 and references within]. However, many of the animal models exhibit responses to infection that are not similar to what has been recorded in humans. In particular, immune enhancement has been reported with a feline infectious peritonitis (FIP) coronavirus (FIP-CoV) vaccine [7], and exacerbated hepatitis was reported when a SARS-CoV spike protein vaccine was expressed from a vaccinia vector in ferrets [8]. These and other potential problems arising from SARS-CoV vaccines need to be examined further.

Ferrets *(Mustela putorius furo)* were first noted as

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Table 1. Histopathological findings in lung tissue of ferrets.

Vaccine, ferret	Bronchiole hyperplasia	Perivascular lymphocyte cuffing
Mock		
41275	+/−	+/−
41219	+/−	+
41247	+	+
50409	+/−	+/−
41195 (no challenge)	−	+/−
FI-SARS		
50441	−	+
41271	+	−
41267	+	+
50421	+/−	+
41151 (no challenge)	−	−

NOTE. Lung disease was scored as follows: no lesions (−), minimal degree of lesions (+/−), mild (+), and moderate (++). FI-SARS, formalin-inactivated severe acute respiratory syndrome–associated coronavirus vaccine.

being susceptible to SARS-CoV infection in 2003 [9], and, although the ferrets did not die of pneumonia, virus was isolated from lung tissue. When a neutralizing monoclonal antibody was administered to ferrets prophylactically, it completely prevented SARS-CoV–induced macroscopic lung disease and significantly reduced viral shedding in pharyngeal secretions [10]. Immunization of ferrets with a modified recombinant vaccinia virus vaccine induced neutralizing antibodies, but it also induced a strong inflammatory response, possibly leading to enhanced hepatitis [8]. These responses were linked to recombinant vaccinia virus expressing SARS-CoV proteins [11]. Ferrets were chosen here as a model to test vaccine safety because of these reports linking enhanced liver disease after vaccination. Ferrets are a well-characterized model for the study of respiratory viruses and have been successfully infected with SARS-CoV [9], although studies have been few and lacking microhistopathological data for lung tissue. It is also unclear from past reports what, if any, coronaviruses the animals were previously exposed to.

Here, we have used coronavirus-free animals and examined the effects of an inactivated vaccine and virus challenge. In addition to analysis of the humoral response, we examined lung tissue of ferrets by microhistopathological analysis. The results of this pilot study will provide a basis for future experiments in the ferret model and demonstrate that an inactivated vaccine shows no evidence of enhanced disease. The results obtained in the ferret model will be a good comparator with the mouse models [12, 13], and this additional animal system will be useful for evaluating SARS vaccine safety and efficacy.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney (Vero E6) cells were used to grow SARS-CoV (Urbani strain) in Dulbecco's modified Eagle medium (DMEM; Biosource) with supplements (10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.5 μg/mL fungizone; Biosource International). Supernatants were collected, pooled, clarified by centrifugation, and stored at −70°C for use as the viral stock. The stock was filtered through a Millex-GS 0.22-

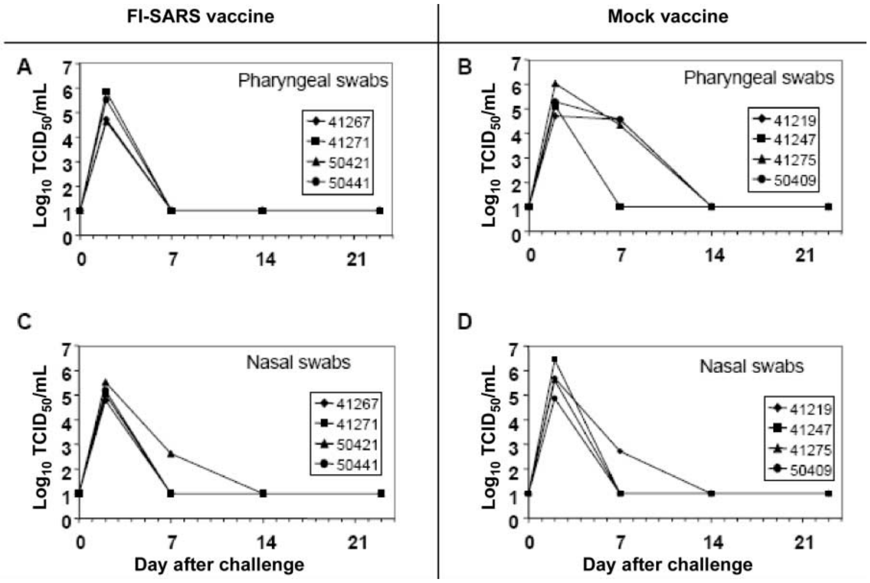

Figure 1. Viral titers in pharyngeal (*A* and *B*) and nasal (*C* and *D*) swabs from ferrets vaccinated with formalin-inactivated severe acute respiratory syndrome (SARS)–associated coronavirus vaccine (FI-SARS) (*A* and *C*) and mock-vaccinated ferrets (*B* and *D*). Virus was collected by use of swabs in Dulbecco's modified Eagle medium and was analyzed by $TCID_{50}$ assay. The limit of viral detection for this assay was 1 $\log_{10}$ $TCID_{50}$/mL.

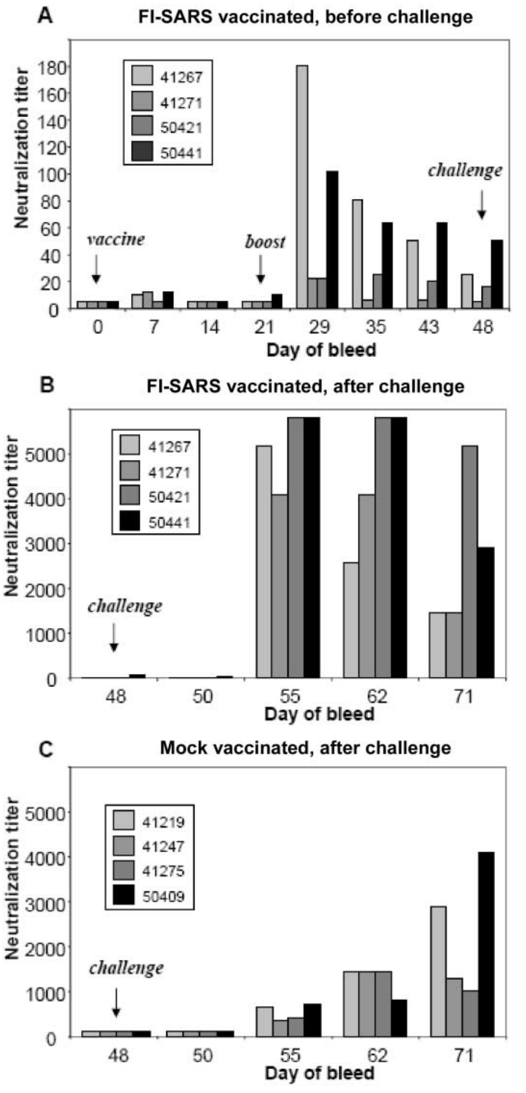

Figure 2. Neutralizing antibody titers for ferrets vaccinated with formalin-inactivated severe acute respiratory syndrome (SARS)–associated coronavirus vaccine (FI-SARS) before *(A)* and after *(B)* challenge and for mock-vaccinated ferrets after challenge *(C)*. Data are expressed as the dilution of serum that prevented cytopathic effect in 50% of the wells and were calculated by the method of Reed and Muench [17]. Results from individual ferrets are shown. Note the scale change between before and after challenge.

μm filter unit (Millipore). The titer (1×10^6 $TCID_{50}$/mL) was determined by a $TCID_{50}$ assay, as described elsewhere [14].

Ferrets. Ten coronavirus-free, 6–20-month-old female ferrets (Harlan) were housed individually for 2 weeks for observation before being vaccinated. Ferrets were housed in a biosafety level (BSL) 2 laboratory until virus challenge in a BSL3 laboratory. Light was managed to delay estrus, and ferrets coming into estrus were given 1 dose of human chorionic gonadotropin (100 IU). Ferrets were monitored daily for care and health. Ferrets were lightly anesthetized with ketamine (5 mg/kg), xylazine (0.5 mg/kg), and acepromazine (0.1 mg/kg) intramuscularly as well as with atropine (0.05 mg/kg) for collection of blood samples (from the jugular vein) and challenge with SARS-CoV. Ferrets were monitored until awakening and thereafter checked to ensure that they were eating, drinking, and behaving normally. Euthanasia was performed with pentobarbitol sodium (390 mg/mL; 1 mL/10 kg of body weight) after initial sedation with ketamine/xylazine/acepromazine, as per the guidelines of the Institutional Animal Care and Use Committee of the Center for Biologics Evaluation and Research, US Food and Drug Administration, and in accordance with the guidelines of the National Institutes of Health Animal Care and Use Committee.

Nasal and pharyngeal swabs. Nasal swabs were obtained with premoistened (DMEM) swabs. The nostrils on ferrets are very small, and samples were collected just a few millimeters into the nose. A laryngoscope was used to view the glottis, and pharyngeal swabs were collected using Dacron-tipped applicators.

Formalin-inactivated virus vaccine and challenge. To generate the vaccine, 5.94 mL of virus stock (1×10^5 $TCID_{50}$/mL) was treated with 60 μL of 3.7% formalin for 48 h at 37°C. The inactivated virus (6 mL) was then dialyzed in 2 L of PBS at room temperature for 48 h. Medium from uninfected Vero cells was treated in the same way to prepare mock vaccine. Vaccine was plated on Vero cells for 7 days to confirm inactivation. Vaccination was done intramuscularly at 0.5 mL per leg without adjuvant. Five ferrets were given the SARS vaccine, and 5 were given the mock vaccine (table 1). Immunization was repeated (boost) after 3 weeks.

The virus challenge (1×10^5 $TCID_{50}$/mL) was split into 2 parts: 0.25 mL was given intratracheally, and 0.25 mL was given intranasally. A laryngoscope was used for the intratracheal portion, which was delivered via a 1-cc syringe with a 22-gauge plastic angiocatheter attached. The intranasal inoculum was delivered to each nostril via a plastic bulb–type pipettor. Four ferrets in each vaccine group were challenged with live SARS-CoV 27 days after the boost (table 1).

Quantitation of viral titer. Viral titers were determined by observing infected Vero E6 monolayers in 24- and 96-well plates by use of a $TCID_{50}$ assay. Ten-fold serial dilutions of viral samples were incubated at 37°C for 4 days and then examined for cytopathic effect (CPE) in infected cells, as described elsewhere [15]. Briefly, CPE of SARS-CoV–infected Vero E6 cells was determined by observing rounded, detached cells in close association with each other. The first dilution of viral sample was a 1:10 dilution, which set the limit of viral detection for this assay at 1 $\log_{10}$ $TCID_{50}$/mL.

Microneutralization assays. Ferret serum was treated at 56°C for 30 min to inactivate complement and then was diluted 1:4 with DMEM. Serial 1:2 dilutions of the serum were made in a 96-well tissue culture plate by adding 125 μL of the serum at a 1:4 dilution to an equal volume of DMEM; this was carried

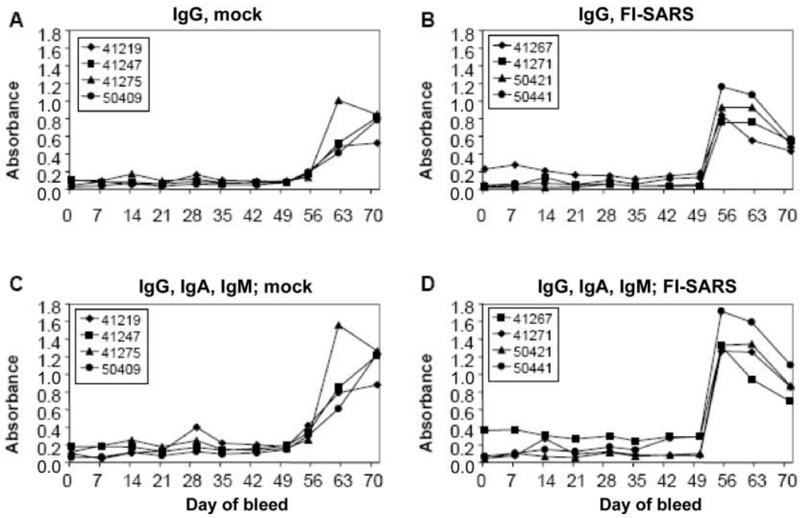

Figure 3. Antibody titers measured by ELISA for IgG alone (*A* and *B*) and for IgG+IgA+IgM (*C* and *D*) in mock-vaccinated ferrets (*A* and *C*) and in ferrets vaccinated with formalin-inactivated severe acute respiratory syndrome (SARS)–associated coronavirus (SARS-CoV) vaccine (FI-SARS) (*B* and *D*). Recombinant spike protein was used to measure ferret antibodies to SARS-CoV.

through a 1:4096 dilution, as described elsewhere [16]. An equal volume of SARS-CoV (100 $TCID_{50}$) was added, and the mixture was incubated for 1 h at room temperature. The mixture was plated onto Vero cell cultures to detect the presence of nonneutralized virus. The dilution of serum that prevented 50% of the CPE in the cell culture wells was reported as the antibody neutralizing titer.

ELISAs. Immuno MaxiSorp plates (Nunc) were coated with 100 μL per well of 2 μg/mL recombinant SARS-CoV spike protein (Imgenex) in 50 mmol/L sodium carbonate coating buffer (Imgenex) at 4°C overnight. Antigen was removed, and the plates were blocked with 200 μL per well of 1% bovine serum albumin (BSA) in Tris-buffered saline with 0.5% Tween (TBS-T) for 2 h at room temperature. Ferret serum was diluted 1:200 in TBS-T with 1% BSA and was added to duplicate wells for incubation at room temperature. The plates were washed with TBS-T. Peroxidase-conjugated goat anti–ferret IgG (Kirkegaard and Perry Laboratories) or goat anti–ferret IgG +IgA+IgM (Rockland) was used to detect bound antibody. Peroxidase substrate solution (KPL) containing 2,2′-azino-di-3-ethylbenzthiazoline-6-sulfonate was used to develop the plates at 405 nm with a Model 450 Microplate Reader (Bio-Rad Laboratories).

Histopathological analysis. Ferrets were euthanized on day 23 after challenge; liver, lungs, and other major organs were fixed in formalin and embedded in paraffin, and sections were stained with hematoxylin-eosin. Selected liver lesions were stained by the Steiner technique.

Ferret health evaluation. A digital thermometer was used to take ferret rectal temperatures. Ferrets were weighed weekly. Ferret blood chemistry analysis was performed with serum drawn on the day of challenge and weekly thereafter, using a Vet Scan II analyzer (Abaxis) with liver-profile rotors.

RESULTS

Mild protection of ferrets from SARS-CoV conferred by inactivated vaccine. Ferrets were inoculated with a mock vaccine or with a formalin-inactivated whole-virus SARS-CoV vaccine. After 2 weeks, a booster immunization was administered, and, 4 weeks later, test ferrets were challenged intratracheally and intranasally with live SARS-CoV. Nasal and pharyngeal swabs were collected 2 days after challenge and weekly thereafter and then incubated in cell culture for titration analyses (figure 1). Two days after challenge, viral titers were similar in the pharynx and nose for both the SARS-CoV vaccine (figure 1*A* and 1*C*) and the mock vaccine (figure 1*B* and 1*D*) groups. By 7 days after challenge, all of the ferrets in the SARS-CoV vaccine group had cleared the virus from the pharynx (figure 1*A*), but 3 of 4 ferrets in the mock vaccine group did not clear virus until day 14 (figure 1*B*). The earlier clearing of the virus from the pharyngeal secretions indicates that the inactivated SARS-CoV vaccine provided immune protection to the ferrets.

To examine the level of immune response provided by the inactivated SARS-CoV vaccine, we used serum to perform neutralization assays. Two of the 4 ferrets that received SARS-CoV vaccine (41267 and 50441) displayed some neutralizing antibodies after the second immunization, which tapered off after 3 weeks (figure 2*A*). The other 2 SARS-vaccinated ferrets showed little or no neutralizing antibody even after the second

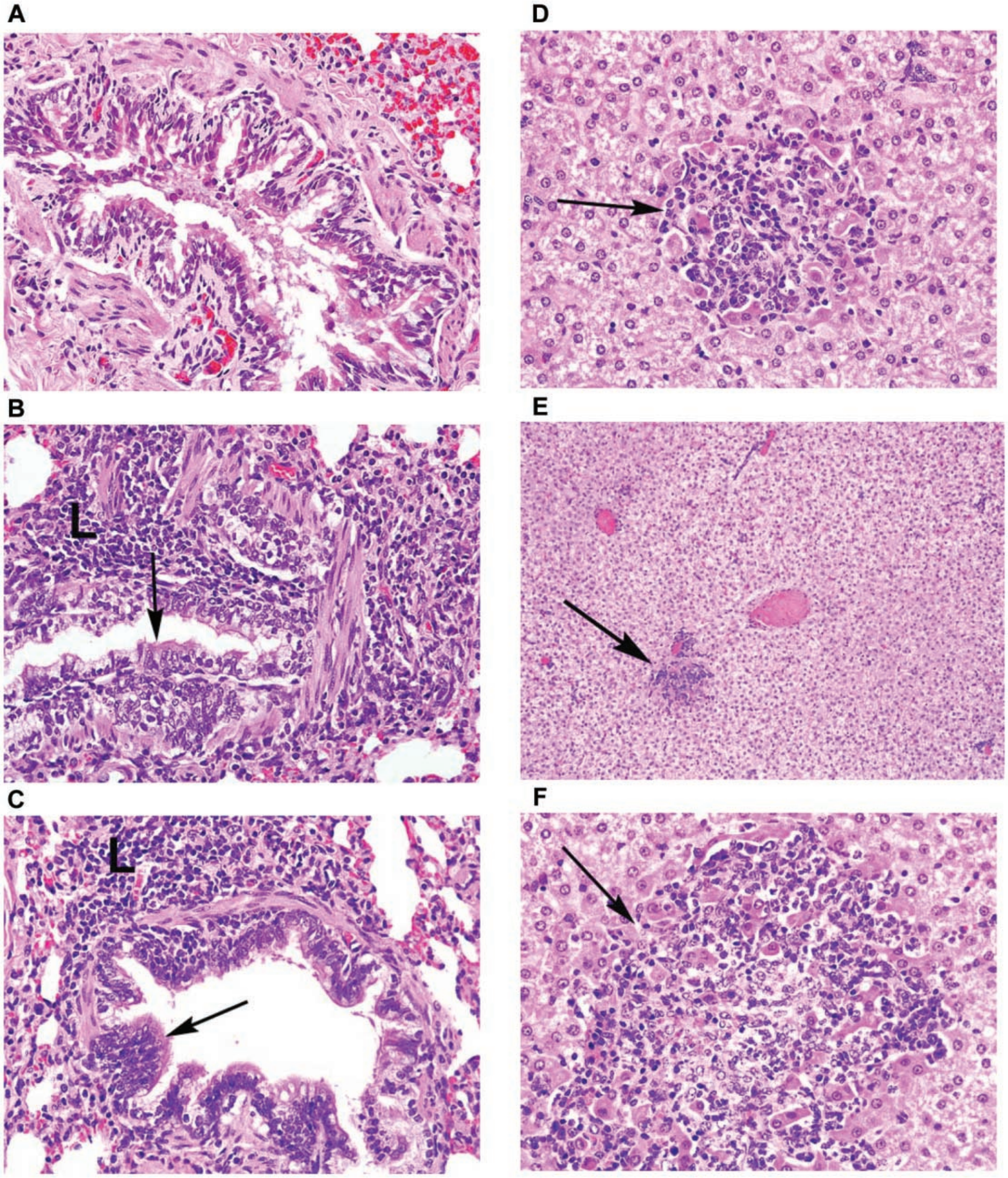

Figure 4. *A,* Normal bronchiole from a control ferret showing normal, uniformly layered epithelium (hematoxylin-eosin [HE] stain; original magnification, ×400). *B,* Bronchiole of ferret given mock vaccine and challenged with virus 23 days earlier showing epithelial hyperplasia *(arrow)* and peribronchiolar inflammation primarily composed of lymphocytes (L) (HE; original magnification, ×400). *C,* Bronchiole of vaccinated, virus-challenged ferret showing focal bronchiolar hyperplasia *(arrow)* and peribronchiolar inflammation primarily composed of lymphocytes (L) (HE; original magnification, ×400). *D,* Liver of ferret given mock vaccine and challenged with virus showing a focal necrotizing inflammatory lesion *(arrow)* (HE; original magnification, ×400). *E,* Liver of vaccinated, virus-challenged ferret showing that the major portion of the section is normal *(middle and right side)* while a small inflammatory focus is seen *(arrow)* (HE; original magnification, ×100). *F,* Liver of vaccinated, virus-challenged ferret with a focal necrotizing inflammatory lesion composed of a mixed population of cells, including lymphocytes and macrophages (HE; original magnification, ×400).

immunization. All 4 ferrets displayed high levels of neutralizing antibody 1 week after challenge, with the titers waning at 3 weeks after infection, concurrent with clearance of the virus (figure 2*B*). The mock-vaccinated ferrets showed no virus neutralization before challenge (data not shown) and increasing neutralization 3 weeks after challenge, but with lower titers than those in the SARS-vaccinated ferrets (figure 2*C*). The 2 mock-vaccinated ferrets that showed high neutralizing titers 3 weeks after infection (41219 and 50409) also had high virus titers at day 14. One mock-vaccinated ferret (41247) cleared the virus

Table 2. Blood chemistry analysis of mock-vaccinated ferrets and of ferrets vaccinated with formalin-inactivated severe acute respiratory syndrome (SARS)–associated coronavirus vaccine (FI-SARS).

	Female ferrets						
Test, vaccine	Observed range	Mean ± SE	Day 0	Day 2	Week 1	Week 2	Week 3
Albumin level, U/L	3.3–4.2	3.8 ± 0.2					
Mock			3.4–4.7	3.2–3.9	3.3–3.8	3.3–4.0	3.5–4.3
FI-SARS			4.1–4.5	3.6–4.1	3.2–4.2	3.9–4.4	3.9–4.8
ALP level, U/L	31–66	44.3 ± 11.3					
Mock			21–48	30–46	29–81	30–41	26–40
FI-SARS			19–28	23–43	23–33	27–35	18–32
ALT level, U/L	78–149	150.3 ± 49.3					
Mock			85–139	121–143	61–163	52–98	63–93
FI-SARS			88–162	90–277	56–88	64–94	79–135
Amylase level, U/L							
Mock			11–20	27–34	28–29	18–24	16–19
FI-SARS			10–17	26–32	21–27	19–23	14–20
Total bilirubin level, mg/dL	0–0.1	ND					
Mock			0.2–0.3	0.2–0.3	0.30	0.30	0.30
FI-SARS			0.2–0.2	0.2–0.3	0.2–0.3	0.2–0.3	0.30
Urea nitrogen level, mg/dL	11–25	33.3 ± 7.6					
Mock			17–33	17–29	22–36	27–30	26–31
FI-SARS			15–21	23–29	19–25	19–31	16–30
Calcium level, mg/dL	7.5–9.9	9.0 ± 0.3					
Mock			9.2–11.8	9.3–10.3	8.9–10.7	8.3–9.7	8.7–10.2
FI-SARS			9.5–10.4	9.0–9.9	9.1–10.7	9.3–10.3	9.0–10.5
Phosphorus level, mg/dL	4.8–7.6	6.7 ± 0.6					
Mock			5.5–7.8	4.8–7.7	5.9–8.6	4.9–6.4	5.7–7.5
FI-SARS			6.1–6.6	5.2–7.1	6.1–9.0	6.2–7.0	5.7–8.2
Creatinine level, mg/dL	0.3–0.8	0.4 ± 0.1					
Mock			0.2–0.5	0.2–0.5	0.4–0.7	0.4–0.6	0.4–0.6
FI-SARS			0.2–0.3	0.2–0.4	0.2–0.5	0.3–0.5	0.4–0.8
Glucose level, mg/dL	99–135	104.9 ± 16.4					
Mock			99–131	118–127	110–122	106–119	101–109
FI-SARS			101–124	107–132	100–114	102–114	76–108
Sodium level, mmol/L	152–164	150.4 ± 1.5					
Mock			149–170	143–150	138–156	137–149	143–149
FI-SARS			148–150	142–150	138–155	150–154	143–154
Potassium level, mmol/L	4.1–5.2	4.9 ± 0.3					
Mock			4.8–6.2	4.6–4.7	4.6–5.3	4.3–4.7	4.4–4.5
FI-SARS			4.4–5.0	4.6–5.0	5.0–5.2	4.4–5.2	4.5–5.4
Total protein level, g/dL	5.0–6.8	6.0 ± 0.5					
Mock			5.9–7.8	5.6–6.3	6.1–6.7	5.6–6.4	5.9–6.7
FI-SARS			6.3–6.7	5.9–6.5	5.0–7.3	6.6–7.0	5.9–7.4
Globulin level, g/dL	1.8–3.1	ND					
Mock			2.1–3.4	2.2–2.5	2.5–3.1	2.0–2.6	2.1–2.4
FI-SARS			2.1–2.3	2.2–2.9	2.7–3.1	2.5–2.8	2.0–2.6
Hemolysis							
Mock			1–2+	1–2+	1–2+	1+	0–2+
FI-SARS			1–3+	2+	1–2+	1–2+	1–2+
Lipemia							
Mock			0–3+	1–3+	1–3+	1–2+	1–2+
FI-SARS			1+	1+	1+	1–2+	1+
Icterus							
Mock			0	0	0	0	0
FI-SARS			0	0	0	0	0

NOTE. Data for study ferrets are ranges or means; observed ranges and mean ± SE values for female ferrets are considered normal [19]. ALP, alkaline phosphatase; ALT, alanine aminotransferase; ND, not done.

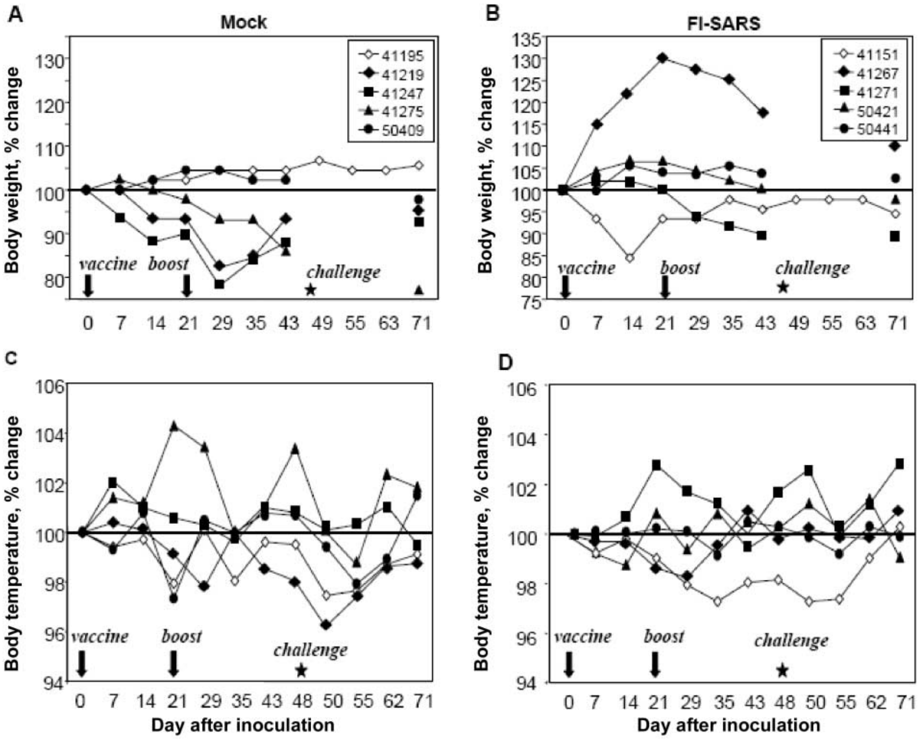

Figure 5. Percent body weight and body temperature change in vaccinated ferrets. Weights were recorded at time zero for mock-vaccinated ferrets *(A)* and ferrets vaccinated with formalin-inactivated severe acute respiratory syndrome (SARS)–associated coronavirus vaccine (FI-SARS) *(B)*. Rectal temperatures were taken using a digital thermometer for mock-vaccinated *(C)* and vaccinated *(D)* ferrets. The percentage of deviation from the starting (100%) weight or temperature for each ferret is shown.

by day 7 (figure 1*B*) and did not have high neutralizing antibody titers (figure 2*C*). The development of neutralizing antibodies was not strongly induced by the vaccine but was significantly induced in all challenged ferrets. This suggests that the vaccine was not immunologically robust.

Characterization of the humoral immune response to vaccination. To further evaluate the potency of the humoral immune response elicited by the SARS-CoV vaccine and infection, we measured virus-specific IgG levels in ferret serum by ELISA. Overall, no significant elevation in spike-specific IgG levels was seen as a result of the vaccine (days 0 and 21), but virus challenge induced a marked elevation in levels of IgG antibodies (figure 3*A* and 3*B*). The mock-vaccinated nonchallenged ferret displayed no antibodies to the SARS-CoV spike protein (data not shown). The SARS-vaccinated ferrets produced higher levels of spike-specific IgG after challenge, and these antibodies were detected 1 week earlier than in the mock-vaccinated ferrets, suggestive of a priming effect of the vaccine. To determine whether this observation was due to the generation of IgM antibodies, IgG+IgA+IgM was measured. The levels of these antibodies were also slightly elevated in the SARS-vaccinated ferrets and were detected earlier than in the mock-vaccinated ferrets (figure 3*C* and 3*D*). This suggests that, although the vaccine generated only a weak antigenic response to the spike protein, it may have primed the immune response, leading to higher levels of other antibodies (figure 1) and faster clearing of the virus.

Pathological findings in lung tissue of vaccinated ferrets. Four mock-vaccinated and 4 SARS-vaccinated ferrets were challenged with SARS-CoV. Two ferrets were used as uninfected control animals. Ferrets were euthanized 23 days after challenge. In the mock-vaccinated unchallenged control ferret (41195), the bronchiolar epithelium appeared normal in thickness. There was no inflammation, nor was bronchial and bronchiolar hyperplasia observed (figure 4*A*). In a mock-vaccinated SARS-CoV–infected ferret (41247), there was focal bronchiolar hyperplasia (figure 4*B*), which was also present in other challenged mock-vaccinated or SARS-vaccinated ferrets (figure 4*C*). The inflammatory cells, mainly lymphocytes, were peribronchiolar (figure 4*B* and 4*C*). In addition, there was perivascular cuffing around a few small blood vessels. Virus was not found in any of the lung tissue by culturing in vitro or by staining sections (data not shown). Bronchial and bronchiolar hyperplasia was evident in 7 of the 8 ferrets that were challenged with virus but was not evident in the unchallenged control ferrets (table 1), suggesting that SARS-CoV infection led to the lesions, not the vaccine. The lymphocyte cuffing also found in 7 of the 8 challenged ferrets suggested a role for the immune system in resolving pulmonary infection (table 1).

Hepatic pathology. All ferret livers appeared normal on gross examination. Histologically, the majority of the livers appeared normal except for foci of necrosis and inflammation in the ferrets exposed to virus. The uninfected, nonvaccinated control ferret did not have these lesions. A few foci of liver-cell necrosis with mononuclear infiltration were observed (figure 4*D*–4*F*). However, this phenomenon is not always a specific feature of viral hepatitis. Other evidence of typical viral hepatitis, such as diffuse hepatic inflammatory lesions, acidophilic bodies, and piecemeal necrosis in the portal tract, was not seen. We observed a larger lesion in the SARS-vaccinated ferret (figure 4*F*) than in the mock-vaccinated ferret (figure 4*D*), but, because virus was not found in the livers, the lesions found histologically were probably from previous viral damage. We checked for the presence of bacteria (by use of Steiner stain) in the hepatic lesions and found none.

Ferret health: blood chemistry. Because elevated levels of alanine aminotransferase (ALT), an indicator of hepatitis, have been observed in human patients with SARS [18] and in vaccinated ferrets [8], we used blood chemistry analysis to evaluate ferret health after vaccination and virus infection. We observed elevated alkaline phosphatase levels in 1 mock-vaccinated ferret (50409) and a slightly elevated ALT level in this same ferret 1 week after challenge (table 2). Normal ranges were observed thereafter. In the SARS-vaccinated group, 1 ferret (41271) had a slightly elevated ALT level on the day of challenge and a highly elevated ALT level 1 week later (table 2), but the level then returned to the normal range. This is consistent with the kinetics of SARS-CoV infection, which is brief, as opposed to a result of an immune response, which is expected to be more protracted. Liver injury, therefore, may not be associated with our weak formalin-inactivated whole-virus vaccine but with SARS-CoV infection in a small percentage of animals.

Ferret body weight and temperature. Ferret 41247 had the most severe lung lesions and also had the largest overall weight loss (22%; figure 5*A*). Three of the 5 ferrets in the mock vaccine group showed some weight loss (mean loss, 15%) after vaccination, with nearly full recovery of body weight on clearance of the virus (day 71). The exception was ferret 41275, which lost >20% of its body weight after viral clearance (figure 5*A*). Two of the SARS-vaccinated ferrets lost weight after vaccination, and 1 recovered to almost normal weight (−5%) after the vaccine boost (figure 5*B*). Overall, the SARS-vaccinated ferrets maintained or gained more weight after challenge, suggesting that the overall health of the vaccinated ferrets was better. One ferret from each vaccine group (41275 and 41271; figure 5*C* and 5*D*) spiked a temperature on the day of boost and challenge, suggesting that body temperature was associated with stress. No significant increase in body temperature was observed in any of the 10 ferrets after vaccination or virus challenge (figure 5*C* and 5*D*).

DISCUSSION

In preparation for a potential SARS-CoV outbreak, a safe and effective vaccine must be available. The pathology of this zoonotic viral infection indicates that pneumocytes are the primary target of infection, resulting in diffuse alveolar damage. To evaluate the safety and efficacy of any potential SARS-CoV vaccine, an animal model is needed that reliably induces severe disease or death. To date, animal models that mimic human SARS-CoV fatality rates are lacking. In fact, no animal infected with SARS-CoV has shown a severe viral pneumonia leading to death, as is seen in human infections. Animal infections are usually characterized by bronchiolar lesions with little or no alveolar involvement. Ferrets have long been used to study influenza virus infection and pathogenesis, and ferrets are a plausible model for the study of SARS-CoV infection and pathogenesis.

In one study, nearly 70% of the human patients infected with SARS-CoV had elevated ALT levels [20]. Additionally, severe hepatitis has been shown to be predictive of poor clinical outcome in patients with SARS [20, 21]. One report demonstrated that ferrets presented mild focal necrotizing and inflammatory liver lesions as a result of SARS-CoV infection [9]. Enhanced liver disease and highly elevated ALT levels were also observed in ferrets that were vaccinated with a recombinant vaccinia virus vaccine expressing the SARS-CoV spike protein [8]. We evaluated the humoral immune response to SARS-CoV immunization and infection and did not observe high ALT levels in ferrets after vaccination with inactivated SARS-CoV or after live virus challenge. This difference may be due to several factors, such as the specific viral strain used, titer of virus challenge, strength of the immune response, specific response to the spike protein, use of female ferrets, or the presence or absence of vaccinia virus components. More experiments are needed to settle these apparent differences.

In our study, lung lesions were mild to minimal in severity and were focal. Most of the lungs were histologically normal, and none of the lesions would probably cause illness or death. The bronchiole and perivascular lesions seem to be related to previous virus exposure. They are similar to lesions found in monkeys [22] and mice [23] days to weeks after exposure. Lesions were not found in all ferret lungs and were not diffuse. The worst lesion was in the lungs of a ferret in the mock vaccine group, consistent with virus-associated, not vaccine-associated, disease. Lesions found in some ferrets included the bronchiolar hyperplasia and perivascular cuffing often found in monkeys and mice with experimental SARS-CoV infection and a lesion not usually reported—focal hepatic inflammation, which was minimal. We did not recover virus from lung or liver tissue, because ferrets were euthanized after the optimal time for virus collection [24].

We used ferrets to evaluate the safety of a weak, inactivated whole-virus vaccine. Antibody-dependent enhancement (ADE) has been observed during FIP-CoV infection [7] and may be part of the pathogenesis of SARS-CoV infection. Disease associated with SARS-CoV infection results from immune-mediated infiltration of the airways by lymphocytes and macrophages. Enhanced disease should be marked by increased viral titer after prior exposure to the virus or virus components. In the case of FIP, the virus gains entry into macrophage cells via neutralizing antibodies to the spike envelope protein, which bind the virus and are internalized by the cell. Macrophages have been shown to be virally infected in patients with SARS, and the concern is that these macrophages provide an additional host substrate in which the virus may grow. Furthermore, early and more robust seroconversion has been associated with more severe disease in patients with SARS, implicating SARS as an immune-mediated disease [25]. If SARS-CoV gains entry to macrophages through antibodies, then vaccines that elicit these responses may not be safe. We purposely designed a weak vaccine that elicited a mild antibody response to test the possibility that low antibody titers are associated with enhanced pulmonary disease as opposed to a strong antibody response, which should be completely protective. We did not see an increase in viral titer in vaccinated ferrets versus mock-vaccinated ferrets, and the vaccine did not induce ADE. This suggests that, under these conditions, an inactivated whole-virus vaccine can provide some measure of protection without the risk of enhanced lung or liver disease. Still needed are kinetic studies to show that infected ferrets are protected from further infection in a dose-dependent manner. Furthermore, the role played by the level of the immune response in ADE needs to be analyzed.

Several animal models have shown promise for use in the evaluation of SARS antivirals, vaccine safety, and efficacy. The most notable studies are those using mice [16, 26, 27] and hamsters [28]. Although the ease of using large numbers of small rodents makes the mouse and hamster models invaluable, the difficulty in performing experiments under BSL3 containment conditions limits the use of larger animals. Ferrets may provide a more convenient alternative to nonhuman primates and an additional model to small rodents. Here, we show that a weak formalin-inactivated vaccine does not enhance disease and that the ferret is a good model for evaluating SARS vaccine safety and efficacy.

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References

1. Lau SK, Woo PC, Li KS, et al. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci USA **2005**; 102:14040–5.
2. Li W, Shi Z, Yu M, et al. Bats are natural reservoirs of SARS-like coronaviruses. Science **2005**; 310:676–9.
3. Woo PC, Lau SK, Yuen KY. Infectious diseases emerging from Chinese wet-markets: zoonotic origins of severe respiratory viral infections. Curr Opin Infect Dis **2006**; 19:401–7.
4. World Health Organizaiton. Epidemic and pandemic alert and response: summary of probable SARS cases with onset of illness from 1 November 2002 to 31 July 2003. Available at: http://www.who.int/csr/sars/country/table2004_04_21/en/index.html. Accessed 24 September 2007.
5. Normile D. Infectious diseases: second lab accident fuels fears about SARS. Science **2004**; 303:26.
6. Deming D, Sheahan T, Heise M, et al. Vaccine efficacy in senescent mice challenged with recombinant SARS-CoV bearing epidemic and zoonotic spike variants. PLoS Med **2006**; 3:e525.
7. Olsen CW, Corapi WV, Ngichabe CK, Baines JD, Scott FW. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J Virol **1992**; 66:956–65.
8. Weingartl H, Czub M, Czub S, et al. Immunization with modified vaccinia virus Ankara-based recombinant vaccine against severe acute respiratory syndrome is associated with enhanced hepatitis in ferrets. J Virol **2004**; 78:12672–6.
9. Martina BE, Haagmans BL, Kuiken T, et al. SARS virus infection of cats and ferrets. Nature **2003**; 425:915.
10. ter Meulen J, Bakker AB, van den Brink EN, et al. Human monoclonal antibody as prophylaxis for SARS coronavirus infection in ferrets. Lancet **2004**; 363:2139–41.
11. Czub M, Weingartl H, Czub S, He R, Cao J. Evaluation of modified vaccinia virus Ankara based recombinant SARS vaccine in ferrets. Vaccine **2005**; 23:2273–9.
12. Stadler K, Roberts A, Becker S, et al. SARS vaccine protective in mice. Emerg Infect Dis **2005**; 11:1312–4.
13. Bisht H, Roberts A, Vogel L, Subbarao K, Moss B. Neutralizing antibody and protective immunity to SARS coronavirus infection of mice induced by a soluble recombinant polypeptide containing an N-terminal segment of the spike glycoprotein. Virology **2005**; 334:160–5.
14. Darnell MER, Subbarao K, Feinstone SM, Taylor DR. Inactivation of the coronavirus that induces severe acute respiratory syndrome, SARS-CoV. J Virol Methods **2004**; 121:85–91.
15. Ksiazek TG, Erdman D, Goldsmith CS, et al. A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med **2003**; 348:1953–66.
16. Subbarao K, McAuliffe J, Vogel L, et al. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome coronavirus in the respiratory tract of mice. J Virol **2004**; 78:3572–7.
17. Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. Am J Hyg **1938**; 27:493–7.
18. Cui HJ, Tong XL, Li P, et al. Serum hepatic enzyme manifestations in patients with severe acute respiratory syndrome: retrospective analysis. World J Gastroenterol **2004**; 10:1652–5.
19. Marini RP, Otto G, Erdman S, Palley L, Fox JG. Biology and diseases of ferrets. In: Fox JG, ed. Laboratory animal medicine. 2nd ed. New York: Academic Press, **2002**:483–518.
20. Chan HL, Kwan AC, To KF, et al. Clinical significance of hepatic derangement in severe acute respiratory syndrome. World J Gastroenterol **2005**; 11:2148–53.
21. Chau TN, Lee KC, Yao H, et al. SARS-associated viral hepatitis caused by a novel coronavirus: report of three cases. Hepatology **2004**; 39: 291–4.

22. McAuliffe J, Vogel L, Roberts A, et al. Replication of SARS coronavirus administered into the respiratory tract of African green, rhesus and cynomolgus monkeys. Virology **2004**; 330:8–15.
23. Roberts A, Paddock C, Vogel L, Butler E, Zaki S, Subbarao K. Aged BALB/c mice as a model for increased severity of severe acute respiratory syndrome in elderly humans. J Virol **2005**; 79:5833–8.
24. Roberts A, Wood J, Subbarao K, Ferguson M, Wood D, Cherian T. Animal models and antibody assays for evaluating candidate SARS vaccines: summary of a technical meeting 25–26 August 2005, London, UK. Vaccine **2006**; 24:7056–65.
25. Lee N, Chan PK, Ip M, et al. Anti-SARS-CoV IgG response in relation to disease severity of severe acute respiratory syndrome. J Clin Virol **2006**; 35:179–84.
26. Yang Z-Y, Kong W-P, Huang Y, et al. A DNA vaccine induces SARS coronavirus neutralization and protective immunity in mice. Nature **2004**; 428:561–4.
27. Kong WP, Xu L, Stadler K, et al. Modulation of the immune response to the severe acute respiratory syndrome spike glycoprotein by gene-based and inactivated virus immunization. J Virol **2005**; 79:13915–23.
28. Buchholz UJ, Bukreyev A, Yang L, et al. Contributions of the structural proteins of severe acute respiratory syndrome coronavirus to protective immunity. Proc Natl Acad Sci USA **2004**; 101:9804–9.